Comparative Toxicity of Selected Organophosphate Insecticides against Resistant and Susceptible Clones of the Greenbug, *Schizaphis graminum* (Homoptera: Aphididae)

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Comparative toxicity of selected organophosphate (OP) insecticides against resistant and susceptible clones of the greenbug, *Schizaphis graminum*, were studied both in vitro and in vivo. Two resistant (OR-1 and OR-2) clones of the greenbug showed marginal to high levels of resistance to all seven OPs tested, ranging from 11- to 327-fold greater than those of a susceptible (OSS) clone. The OR-1 clone showed lower levels of resistance to phenyl (parathion and parathion-methyl) and heterocyclic (chlorpyrifos) OPs than to aliphatic OPs (dimethoate, omethoate, disulfoton, and demeton-S-methyl), whereas the OR-2 clone showed a rather broad spectrum of resistance to nearly all OP insecticides examined. In vitro inhibition of acetylcholinesterase (AChE) using six selected OP oxon analogues showed that alterations of AChE were involved in resistance to all OP compounds examined in both the OR-1 and OR-2 clones. Although the levels of insensitivity of AChE to these OPs were relatively low, ranging from 1.1- to 3.8-fold, the insensitivity spectrum of AChE to different OPs was rather broad. The general esterase activity in the OR-1 and OR-2 clones was 1.3−8.4-fold higher than that in the OSS clone, depending on the substrates used. The AChE activity in both the OR-1 and OR-2 clones was 1.8-fold higher than that in the OSS clone. High resistance levels of the OR-2 clone to phenyl and heterocyclic OPs appeared to be associated with the ability of the esterases to hydrolyze β-naphthyl acetate and more hydrophobic substrates.

Keywords: *Organophosphate resistance; acetylcholinesterase; general esterases; greenbug; Schizaphis graminum*

INTRODUCTION

The greenbug, *Schizaphis graminum* (Rondani), is one of the most important insect pests of small grains and sorghum throughout the world (Metcalf and Metcalf, 1993). Insecticide applications provided effective control against its infestation in the United States until the mid 1970s, when resistance to organophosphate (OP) insecticides developed (Peters et al., 1975; Teetes et al., 1975). Since then, several incidences of control failures with most OPs registered against greenbugs have been documented (Archer and Bynum, 1978; Chang et al., 1980; Sloderbeck et al., 1991; Archer et al., 1994; Shufran et al., 1997).

Mechanisms of OP resistance in the greenbug have been investigated in relative detail. Biochemical studies have shown that increased esterase activity plays a major role in conferring OP resistance (Siegfried and Ono, 1993a,b; Ono et al., 1994a). Two OP-resistant clones, OR-1 and OR-2, possess pattern 1 (or type I) and pattern 2 (or type II) esterases, respectively, based on the esterase banding patterns on native polyacrylamide gels (Ono and Siegfried, 1994; Shufran et al., 1996; Zhu and Gao, 1998). Increased activity of the pattern 1 esterases has been demonstrated to be the result of gene amplification (Siegfried et al., 1997; Ono et al., 1999). However, increased activity of the pattern 2 esterases is very likely due to alterations of the gene structure because the amount of esterase expressed in immunological assays is the same as that in the susceptible greenbugs (Siegfried et al., 1997).

Altered acetylcholinesterase (AChE) also has been reported to contribute to overall OP resistance in the greenbug (Siegfried and Ono, 1993a,b; Zhu and Gao, 1999). The AChE from the OP-resistant greenbugs has shown not only reduced sensitivity to inhibition by OPs but also increased catalytic activity toward the model substrate acetylthiocholine (Zhu and Gao, 1999). Recently, we have identified a new OP-resistant clone (OR-0) that showed significant levels of resistance to many OP insecticides but normal levels of general esterase activity (Zhu et al., unpublished data). Our preliminary study did not indicate any significant difference in either cytochrome P450 *O*-demethylase activity or glutathione *S*-transferase activity between the OSS and OR clones. These findings strongly suggested that altered AChE played an important role in conferring OP resistance in the greenbug, particularly in the OR-0 clone.

Although OP resistance in the greenbug is widespread, we know very little about resistance spectra to commonly used OP insecticides. Furthermore, we know nothing about the relationship between the in vivo toxicity of OP insecticides and the in vitro potency in inhibition of AChE. The objectives of this study were to (1) examine the resistance spectra of two previously identified OP-resistant clones of the greenbug, (2) evaluate OP resistance in relation to the potency of OP compounds from in vitro inhibition of AChE, and (3) investigate the status of cross-resistance in relation to

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OP structures. Elucidation of the resistance spectrum and the status of cross-resistance among different OP insecticides will greatly help us not only understand the significance of different resistance mechanisms in conferring resistance to insecticides with different chemistry but also make knowledge-based selections of insecticides for greenbug control and resistance management in the field.

EXPERIMENTAL PROCEDURES

Greenbug Clones. An organophosphate-susceptible strain (OSS) of the greenbug was established from sorghum plants in the greenhouse at Kansas State University in 1996; the source of these greenbugs was unknown. Two resistant strains (OR-1 and OR-2), possessing pattern I and II esterases, respectively, were provided by Blair D. Siegfried at the University of Nebraska, Lincoln, NE. All of the greenbug clones were isolated from their corresponding strains and were maintained on the stems of 6−8-week-old sorghum plants supplied with water in 2-L flasks covered with a nylon screen at ~23 °C. The stems were replaced about every 5 days (Zhu and Gao, 1999). The origins of the two resistant clones were described previously in detail (Ono et al., 1994b). For insecticide bioassays, each clone was mass reared on whole sorghum plants at 25 ± 2 °C in growth chambers with a photoperiod of 16:8 h (L/D). The sorghum hybrid NC+ 160 (susceptible to biotype I greenbugs) was used.

Chemicals. Acetylthiocholine iodide (ATC), bicinchoninic acid (BCA) solution, fast blue salt BN (*o*-dianisidine, tetrazotized), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), α-naphthol, α-naphthyl acetate (α-NA), *β*-naphthyl acetate (*β*-NA), α-naphthyl butyrate (α-NB), α-naphthyl propionate (α-PN), paraoxon (diethyl *p*-nitrophenyl phosphate), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorpyrifos [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate], chlorpyrifos-oxon (diethyl-3,5,6-trichloro-2-pyridinyl phosphate), demeton S {*O*,*O*-diethyl-*S*-[2-(ethylthio)ethyl] phosphorothioate}, demeton-S-methyl (*S*-2-ethylthioethyl *O*,*O*-dimethyl phosphorothioate), dimethoate [*O*,*O*-dimethyl-*S*-(*N*-methylcarbamoylmethyl) phosphorodithioate], disulfoton {*O*,*O*-diethyl-*S*-[2-(ethylthio)ethyl] phosphorodithioate}, methyl paraoxon (dimethyl-*p*-nitrophenyl phosphate), omethoate [*O*,*O*-dimethyl-*S*-(*N*-methylcarbomoylmethyl) phosphorothioate], and parathion (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) were purchased from Chem Service (West Chester, PA). Parathion-methyl (*O*,*O*-dimethyl-*O*-*p*-nitrophenyl phosphorothioate) was supplied by Cheminova Agro (Lemvig, Denmark). Bovine serum albumin (BSA) was purchased from Bio-Rad Laboratories (Hercules, CA).

Insecticide Bioassay. The toxicities of seven OPs (Figure 1) to the three clones of the greenbug were assayed according to the method of Zhu and Gao (1998) with some modifications. The selection of these OPs was based on the diversity of their chemical structures and the availability of their oxon analogues that were used in the AChE in vitro inhibition study. A sample of 20 apterous adult or last instar nymph greenbugs was placed in an 8-mL glass sample vial coated internally with a technical grade OP insecticide in a total of 250 μL of acetone using a Roto-Torque rotator (Cole-Parmer Instrument Co., Inc, Vernon Hills, IL). Five to seven different concentrations of the insecticide, each with four replicates, were used for each bioassay. Mortality was assessed after the treated greenbugs were maintained at 25 ± 1 °C for 8 h. The criterion for death was that an aphid moved <7 mm on filter paper within 30 s when its legs were probed with a soft camel-hair brush. The insecticide bioassay data were analyzed for χ^2, LC_{50} values, and their 95% confidence intervals (95% CI) using probit analysis (PROC PROBIT, SAS Institute, 1996). The resistance ratios and their 95% CIs were calculated according to the method of Roberston and Preisler (1992).

Determinations of AChE Activity, Esterase Activity, and Protein Contents. Ten apterous adults were homogenized in 0.45 mL of ice-cold 0.1 M phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100 for AChE or 0.3% (v/v) Triton X-100 for general esterase assays. After the homogenates were centrifuged at 15000*g* for 15 min at 4 °C, the supernatants were transferred to new tubes and diluted with 0.1 M phosphate buffer (pH 7.0). The AChE activity was measured according to the method of Ellman et al. (1961) with some modifications by Zhu and Gao (1999). General esterase activity was measured according to the methods of Zhu and Gao (1998) using a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA). Acetylthiocholine (ATC) was used as a substrate for AChE, and α-NA, *β*NA, α-NB, and α-NP were used for general esterases. Protein concentrations were determined according to the BCA method (Smith et al., 1985) using BSA as a standard. The measurement was performed using the microplate reader at 560 nm (Zhu and Gao, 1999). All enzyme activities were compared among the three greenbug clones using LSD multiple comparisons (PROC GLM, SAS Institute, 1996).

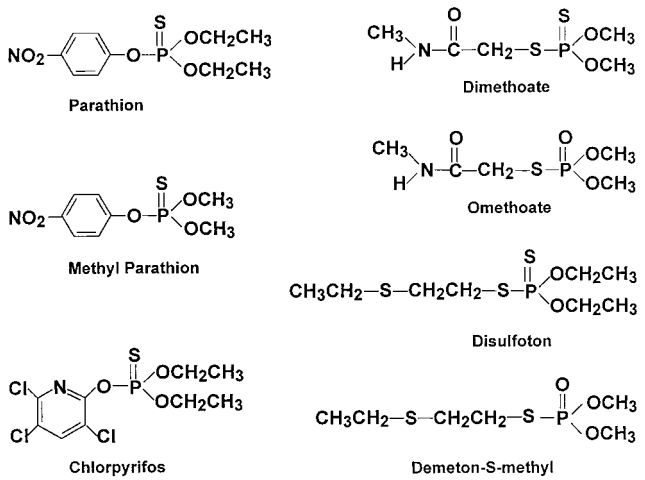

Figure 1. Chemical structures of organophosphate insecticides (OPs) used in the bioassay: (phenyl OPs) parathion and parathion-methyl; (heterocyclic OP) chlorpyrifos; (aliphatic OPs) dimethoate, omethoate, disulfoton, and demeton-S-methyl.

In Vitro Inhibition of AChE. Six OPs were used to determine bimolecular rate constants (k_i). All OPs were dissolved in acetone as stock solutions. The acetone concentration was maintained below 1% in final inhibition reactions. One hundred apterous adults or last-instar nymphs from each clone were homogenized in 1.8 mL of ice-cold 0.1 M phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100. After the homogenates were centrifuged at 15000*g* for 15 min at 4 °C, the supernatants were transferred to new tubes and diluted with 0.1 M phosphate buffer (pH 7.0). The assay procedure was the same as previously described (Zhu and Clark, 1995; Gao et al., 1998). Briefly, 10 μL of each of six different concentrations of OPs was mixed with 10 μL of diluted enzyme using a multichannel pipet and preincubated for 2 min at 25 °C. The residual AChE activity was measured immediately after 180 μL of ATC and DTNB solution was added to the inhibition mixture. The final concentrations of ATC and DTNB in the reaction mixture were 0.50 and 0.04 mM, respectively. Bimolecular rate constants (k_i) were calculated according to the method of Aldridge and Davison (1952). The k_i values were compared among the three greenbug clones using LSD multiple comparisons (PROC GLM, SAS Institute, 1996).

RESULTS AND DISCUSSION

Resistance Spectrum to Selected OP Insecticides. Our studies indicated that the resistance levels in the greenbug were related significantly to the structures of the OP insecticides (Figure 1). Both the OR-1 and OR-2 clones showed marginal to very high levels of resistance to all seven OPs examined, ranging from

Table 1. Susceptibility of Organophosphate-Susceptible (OSS) and -Resistant (OR-1 and OR-2) Clones of the Greenbug to Seven Selected Organophosphate Insecticides

insecticide	clone	*n*	slope (SE)	$P > \chi^2$ [a]	LC_{50} (95% CI),[b] μg/mL	RR (95% CI)[c]
parathion	OSS	449	1.08 (0.13)	0.11	0.20 (0.18−0.22)	
	OR-1	441	0.60 (0.10)	0.12	8.07 (6.88−9.56)	40.8 (33.8−49.2)*
	OR-2	620	1.07 (0.15)	1.00	64.76 (60.35−70.45)	326.9 (288.7−370.1)*
parathion-methyl	OSS	357	1.17 (0.085)	0.06	0.031 (0.019−0.046)	
	OR-1	350	1.03 (0.21)	0.07	0.34 (0.16−0.55)	11.1 (8.7−14.1)*
	OR-2	702	1.10 (0.12)	0.43	1.27 (1.18−1.35)	41.3 (34.7−49.1)*
chlorpyrifos	OSS	536	1.32 (0.17)	0.16	0.036 (0.034−0.039)	
	OR-1	428	1.26 (0.14)	0.06	0.39 (0.30−0.49)	10.6 (9.1−12.3)*
	OR-2	658	0.81(0.045)	0.21	1.96 (1.76−2.17)	53.8 (47.3−61.2)*
dimethoate	OSS	551	1.00 (0.15)	0.05	0.37 (0.31−0.44)	
	OR-1	549	1.15 (0.13)	0.09	7.57 (6.48−8.87)	20.5 (17.6−23.8)*
	OR-2	487	1.44 (0.23)	0.16	6.25 (5.79−6.69)	16.9 (14.8−19.3)*
omethoate	OSS	823	0.76 (0.071)	0.08	0.21 (0.18−0.24)	
	OR-1	410	2.39 (0.26)	0.07	8.01 (7.12−9.04)	38.9 (34.0−44.6)*
	OR-2	579	1.49 (0.20)	0.37	6.28 (5.93−6.66)	30.6 (26.8−34.8)*
disulfoton	OSS	544	0.96 (0.096)	0.32	0.16 (0.15−0.17)	
	OR-1	441	1.87 (0.32)	0.73	6.37 (6.01−6.72)	39.6 (35.8−43.8)*
	OR-2	436	1.11 (0.19)	0.10	15.1 (13.9-16.5)	93.7 (83.2−105.6)*
demeton-S-methyl	OSS	466	1.03 (0.20)	0.82	0.065 (0.061−0.068)	
	OR-1	503	0.34 (0.042)	0.07	3.54 (3.29−3.80)	54.6 (50.7−58.7)*
	OR-2	506	3.08 (0.31)	0.63	3.50 (3.39−3.61)	54.0 (50.7−57.6)*

[a] The value of $P > \chi^2$ larger than or equal to 0.05 indicates a significant fit between the observed and expected regression lines. [b] The LC_{50} values are expressed in micrograms of active ingredient of insecticide per milliliter of acetone and their 95% confidence intervals (95% CI). [c] RR, resistance ratio = LC_{50} of OR/LC_{50} of OSS for a given insecticide. The ratio followed by an asterisk (*) indicates a statistical significance of the resistance based on the non-overlapping 95% CIs of their LC_{50} values between the OSS and OR clones.

11- to 327-fold, compared with those of the OSS clone (Table 1). The lowest resistance levels occurred for parathion-methyl and chlorpyrifos in the OR-1 clone (both 11-fold) and for dimethoate in both the OR-1 (21-fold) and OR-2 (17-fold) clones. Generally, the OR-1 greenbugs showed more distinctive structure-resistance relationships than the OR-2 greenbugs did. Resistance levels to a phenyl (parathion-methyl) and a heterocyclic (chlorpyrifos) OP were relatively low compared with those to the aliphatic OPs (demeton-S-methyl, disulfoton, omethoate, and dimethoate) in the OR-1 clone, whereas the resistance spectrum was rather broad and included all subclasses of OPs in the OR-2 clone.

Our results also indicated that all of the greenbug clones, regardless of their resistance status, were more susceptible to a phenyl (parathion-methyl) and a heterocyclic (chlorpyrifos) OP than to aliphatic OPs. Although parathion and parathion-methyl have very similar structures (Figure 1) and the former is generally more toxic, parathion-methyl appears to be a better control agent for both the OP-susceptible (i.e., OSS) and -resistant (i.e., OR-1 and OR-2) greenbugs. This notion is based on both relatively low LC_{50} values of parathion-methyl for all greenbug clones examined and relatively low levels of resistance to this compound in both the OR-1 and OR-2 clones (Table 1).

Resistance in Relation to in Vitro Inhibition of AChE by OP Compounds. Our results indicated that AChE from either the OR-1 or OR-2 clones was significantly less sensitive ($P < 0.05$) than that from the OSS clone to inhibition by all six OP oxon forms or analogues, including chlorpyrifos oxon, demeton S, demeton-S-methyl, paraoxon-methyl, paraoxon, and omethoate (Figure 2). However, the insensitivity levels of AChE from the two OR clones to these OPs were relatively low, ranging from 1.1- to 3.8-fold, based on the bimolecular rate constant (k_i) (Table 2). Our results clearly indicated that reduced sensitivity of AChE was involved in conferring resistance to all of the OP insecticides examined ($P < 0.05$). Furthermore, the potencies of different OP compounds showed marked differences in in vitro inhibition of AChE from the greenbug. For example, chlorpyrifos oxon exhibited the highest potency (k_i ranged from 1.03×10^7 to 1.31×10^7 M^{-1} min^{-1}), whereas demeton S showed the lowest (k_i ranged from 2.62×10^2 to 3.45×10^2 M^{-1} min^{-1}). Our results generally suggest that phenyl and heterocyclic OPs are

Table 2. Bimolecular Rate Constants (k_i) of Six Organophosphate Compounds in the Inhibition of AChE from Organophosphate-Susceptible and -Resistant Clones of the Greenbug

organophosphate	greenbug clone	$k_i \pm$ SE[a] (M^{-1} min^{-1})	ratio of k_i (OSS/OR)
chlorpyrifos oxon	OSS	$(1.31 \pm 0.05) \times 10^7$ A	
	OR-1	$(1.03 \pm 0.04) \times 10^7$ C	1.3
	OR-2	$(1.16 \pm 0.04) \times 10^7$ B	1.1
paraoxon-methyl	OSS	$(2.90 \pm 0.06) \times 10^5$ A	
	OR-1	$(1.35 \pm 0.07) \times 10^5$ B	2.1
	OR-2	$(1.30 \pm 0.07) \times 10^5$ B	2.2
paraoxon	OSS	$(1.42 \pm 0.02) \times 10^5$ A	
	OR-1	$(0.81 \pm 0.03) \times 10^5$ B	1.8
	OR-2	$(0.72 \pm 0.01) \times 10^5$ C	2.0
demeton-S-methyl	OSS	$(6.04 \pm 0.20) \times 10^4$ A	
	OR-1	$(4.11 \pm 0.08) \times 10^4$ B	1.5
	OR-2	$(4.35 \pm 0.12) \times 10^4$ B	1.4
omethoate	OSS	$(4.62 \pm 0.09) \times 10^3$ A	
	OR-1	$(1.24 \pm 0.08) \times 10^3$ B	3.8
	OR-2	$(1.33 \pm 0.11) \times 10^3$ B	3.5
demeton S	OSS	$(3.45 \pm 0.05) \times 10^2$ A	
	OR-1	$(2.62 \pm 0.08) \times 10^2$ B	1.3
	OR-2	$(2.68 \pm 0.06) \times 10^2$ B	1.3

[a] Values are the means ± standard errors of at least five determinations ($n \geq 5$). Means followed by the same letter are not significantly different for a given compound (LSD, $P < 0.05$).

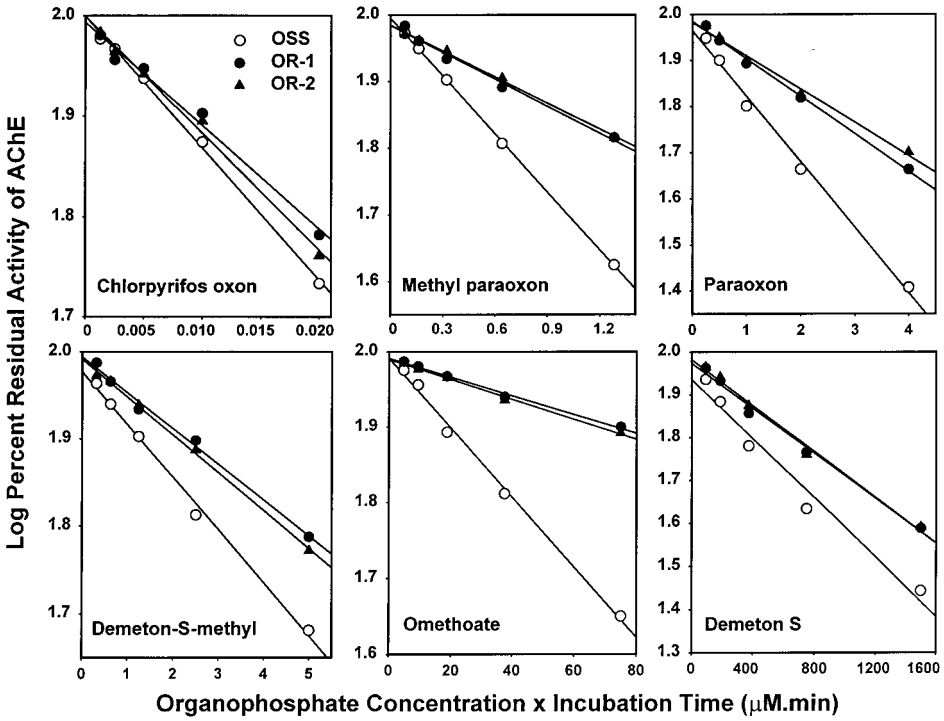

Figure 2. In vitro inhibition of AChE from greenbugs by organophosphate oxon analogues. After the enzyme was preincubated with an organophosphate inhibitor for 2 min at 25 °C, the percentage of residual AChE activity was determined against a control in the absence of the inhibitor. Each point represents the mean of at least five determinations ($n \geq 5$). The correlation coefficients (r) of all linear regressions are >0.979 ($P < 0.01$).

Table 3. Comparisons of Specific Activities (Nanomoles per Minute per Milligram of Protein) of AChE and General Esterases in the Organophosphate-Susceptible and -Resistant Clones of the Greenbug[a]

greenbug clone	general esterases				
	AChE	α-NA	β-NA	α-NB	α-NP
OSS	25.5 ± 1.5 A	21.8 ± 0.5 A	114.3 ± 4.7 A	35.2 ± 1.7 A	37.2 ± 0.7 A
OR-1	46.8 ± 2.2 B	51.6 ± 1.0 B	147.6 ± 8.8 A	50.4 ± 2.2 B	73.8 ± 5.2 B
OR-2	46.5 ± 1.8 B	72.4 ± 0.7 C	721.6 ± 81.9 B	295.5 ± 4.3 C	250.2 ± 4.1 C

[a] Results are presented as the mean ± SE ($n = 5$). Means followed by the same letter are not significantly different among three greenbug clones (LSD, $P < 0.05$).

more potent than the aliphatic OPs in the inhibition of AChE from the greenbug.

However, our study did not show any clear relationship between the OP structure and the degrees of reduced sensitivity to inhibition by these compounds among different greenbug clones, suggesting that the spectrum of low levels of insensitivity of AChE to different OPs is rather broad. These findings explain at least partially why the resistance spectrum to different OP insecticides is so broad in the greenbug. Our results are significantly different from those of some previous studies on the green peach aphid (*Myzus persicae*) (Moores et al., 1994), the Colorado potato beetle (*Leptinotarsa decemlineata*) (Zhu and Clark, 1995), and the lesser grain borer (*Rhyzopertha dominica*) (Guedes et al., 1997). In all of those studies, the degree of reduced sensitivity of AChE was found to be rather dependent on insecticide structure. In certain cases, negative cross-insensitivity of AChE to different OP compounds was found (Zhu and Clark, 1995; Guedes et al., 1997).

Insecticide Structures and Mechanisms of OP Resistance. Previous studies have indicated that increased esterase activities in OR-1 and OR-2 clones were the predominant mechanisms for OP resistance (Siegfried and Ono, 1993a,b; Shufran et al., 1996; Zhu and Gao, 1998). In the OR-1 clone, this mechanism may be more important in conferring resistance to aliphatic OPs because its resistance levels to aliphatic OPs were generally higher than those to heterocyclic (chlorpyrifos) and phenyl (parathion-methyl) OPs (Table 1). In contrast, the OR-2 clone appeared to have a broader resistance spectrum to nearly all subclasses of OPs examined. Apparently, the high resistance levels to phenyl and heterocyclic OPs in the OR-2 clone were associated with the ability of its esterases to hydrolyze β-NA and more hydrophobic substrates (Table 3).

The low levels of insensitivity of AChE in the OR-1 and OR-2 clones suggested that the reduced insensitivity of AChE might not play a major role in conferring overall OP resistance in these greenbug clones. However, altered AChE may contribute significantly to the overall resistance in the OR-1 and OR-2 clones through a secondary mechanism. Combinations of a metabolic and/or sequestration mechanism and target site insensitivity may potentiate each other, resulting in high levels of resistance as documented in other insects (Zhu and Brindley, 1992). Furthermore, the AChE of the OR clones was about twice as active as that of the OSS clone (Table 3). This increased activity may contribute further to the resistance by compensating the inhibited AChE activity and by keeping an adequate functional titer of AChE in the synaptic regions of the nervous system.

Cross-Resistance in Relation to OP Structures. Our study generally showed a cross-resistance among all OP compounds examined in both the OR-1 and OR-2 clones. However, the levels of cross-resistance varied significantly among insecticides and between greenbug clones (Table 1). For example, both the OR-1 and OR-2 clones were markedly cross-resistant to both disulfoton and demeton-S-methyl (Table 1), whereas the cross-resistance to parathion-methyl and chlorpyrifos in the OR-1 clone was marginal (11-fold). On the other hand, the OR-2 greenbugs showed a broader spectrum of cross-resistance to different OPs compared with that of the OR-1 clone. However, the OR-2 greenbugs showed only a low level of resistance to dimethoate (17-fold).

The significant cross-resistance between disulfoton and demeton-S-methyl in both the OR-1 and OR-2 clones of the greenbug may be explained by the structural similarity of these insecticides (Figure 1). In fact, greenbug control failures in the field were first noticed with disulfoton in the 1970s in Texas, Oklahoma, and South Dakota, after a long and intensive use of this compound for greenbug control (Peters et al., 1975; Teetes et al., 1975; Chang et al., 1980). Although we do not know whether the greenbug clones that we tested in this study represent the field populations in which disulfoton resistance was identified originally, demeton-S-methyl has not been used widely for greenbug control in the field. Therefore, the cross-resistance between disulfoton and demeton-S-methyl in both the OR-1 and OR-2 clones appears to be due to their similar chemical structures. Further study is required to understand how different esterases in the OR-1 and OR-2 clones of the greenbug confer cross-resistance among the chemically related and among the chemically unrelated OP insecticides.

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